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Teaching Cases

# Complex *APC* germline mutation associated metaplasia and intraepithelial neoplasia (CAM-IEN) of the gallbladder

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ABSTRACT

Preneoplasic and neoplastic changes of the gallbladder of patients with a familial adenomatous polyposis (FAP) are rare, and very little is known about their incidence in patients with an attenuated FAP. We herein report on a unique case of a woman with an attenuated FAP who shows eight distinct, partially preneoplastic differentiation patterns within the gallbladder mucosa, which are: (1) regular gallbladder epithelium, (2) low grade biliary intraepithelial neoplasia, (3) papillary adenoma, (4) Paneth cell metaplasia, (5) goblet cell metaplasia, (6) pancreatic metaplasia, (7) pseudopyloric metaplasia, and (8) neuroendocrine differentiation. Moreover, this is the first case of a *KRAS* mutation in a gallbladder adenoma of a patient with an *APC* germline mutation, which is highly suggestive of an early event of malignant transformation. As a consequence of our findings, clinicians should draw special attention to the gallbladder of FAP patients, and a simultaneous protective cholecystectomy of FAP patients, which undergo colectomy and show conspicuous changes of the gallbladder mucosa, should be performed in these patients in order to eliminate the risk of a synchronous or metachronous gallbladder neoplasia.



## Introduction

Familial adenomatous polyposis (FAP) is an autosomal dominant inherited polyposis syndrome that is caused by a mutation of the adenomatous polyposis coli (*APC*) gene. It comes along not only with a large number of colorectal adenomas and a highly increased risk for the development of colorectal cancer, but also with an association with extra-colorectal tumors, as it is referred to as Gardner's syndrome. Extracolonic benign and malignant tumors and their preliminary stages occur mainly in the stomach, skin and soft tissue, but, although rare, also *APC* mutation associated neoplasms of the gallbladder are described [1]. Until now, only 11 cases of Gardner's syndrome associated gallbladder adenomas, respectively, six cases of gallbladder adenocarcinomas have been reported, and nothing is known about other precancerous lesions or simultaneous gene mutations [2].

Patients with attenuated FAP do not fulfill the complete criteria for FAP, as they have less than 100 colorectal polyps. Extracolonic manifestations are rare and mainly restricted to gastric polyps, and nothing is described about epithelial alterations of the gallbladder within these patients.

Here, we report for the first time on a case of a woman with an attenuated FAP who shows eight distinct differentiation patterns of the gallbladder mucosa, which include a wide range of precancerous lesions. We found no other name in the literature for this complex metaplastic and dysplastic lesion that we named "**c**omplex ***A***PC germline mutation associated **m**etaplasia and **i**ntra**e**pithelial **n**eoplasia (CAM-IEN) of the gallbladder" and that is a novel carcinogenesis model of the gallbladder, with the possibility to gain new insights into tumor biology.

## Clinical history

A 53-year-old woman with a previously known attenuated FAP and a confirmed *APC* germline mutation (c.4788delC:p.Gin1596Argfs*54) underwent subtotal colectomy with simultaneous cholecystectomy for cholecystolithiasis. The examination of the colectomy specimen revealed multiple residuals of afore endoscopically removed colorectal adenomas with low grade intraepithelial neoplasia according to the WHO-classification. The gallbladder measured $89 \times 28 \times 25$ mm and contained multiple gallstones with a size of up to 17 mm. The gallbladder wall measured 4 mm in maximum thickness. The

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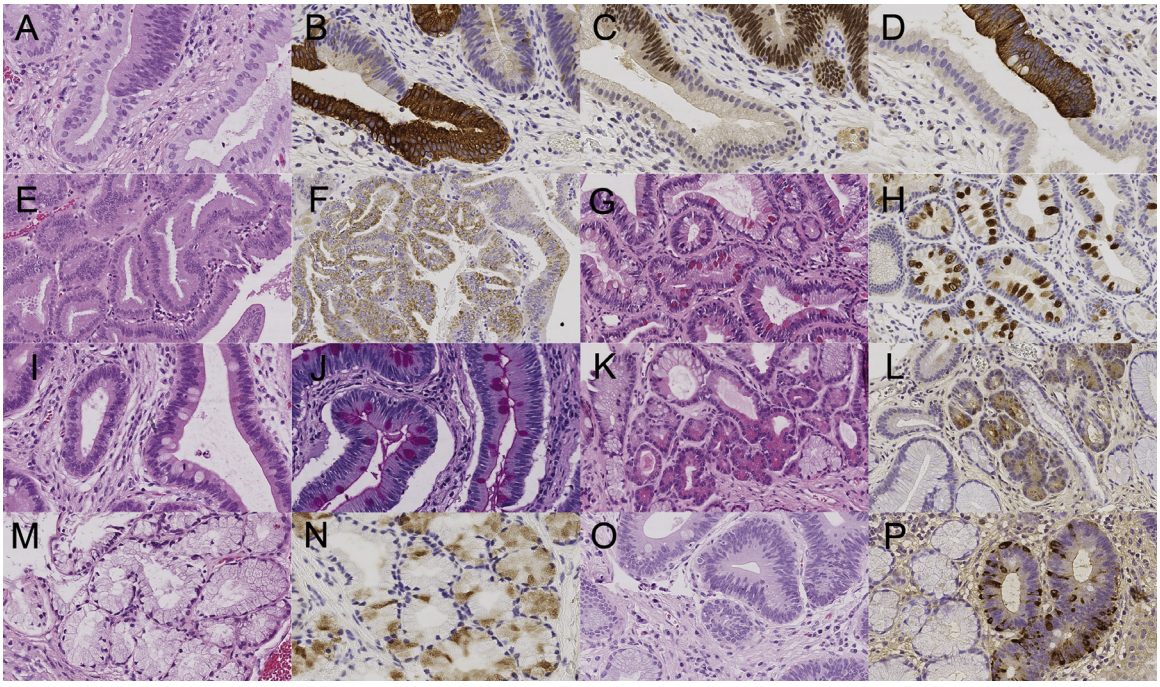


**Fig. 1.** The eight distinct differentiation patterns of the gallbladder mucosa.
The regular gallbladder epithelium merges abruptly into a low grade biliary intraepithelial neoplasia (A–D). The regular gallbladder epithelium is immunohistochemically positive for cytokeratin 7 (B) and negative for CDX2 (C) and cytokeratin 20 (D), whereas the biliary intraepithelial neoplasia is vice versa negative for cytokeratin 7 (B) and positive for CDX2 (C) and for cytokeratin 20 (D). The other differentiation patterns are: Papillary adenoma with an overexpression of LGR5 (E, F); mucin 2-positive Paneth cell metaplasia (G, H); periodic acid-Schiff reaction positive goblet cell metaplasia (I, J); trypsin-positive pancreatic metaplasia (K, L); pseudopyloric metaplasia (M) that is positive for trefoil factor 2 (N); neuroendocrine differentiated cells (O) that express synaptophysin (P). Hematoxylin and eosin (A, E, G, I, K, M and O); cytokeratin 7 (B); CDX2 (C); cytokeratin 20 (D); LGR5 (F); mucin 2 (H); periodic acid-Schiff reaction (J); trypsin (L); trefoil factor 2 (N); synaptophysin (P). Original magnifications ×400 (A–F, I, J and M-P) and ×300 (G, H, K and L).

specimen showed no macroscopic abnormalities, apart from a partial plane polypoid mucosa of 8 mm in diameter.

## Material and methods

### *Immunohistochemical analysis*

Serial sections of 2.5 μm thickness were obtained from formalin-fixed and paraffin-embedded tissue specimens. Immunohistochemical stainings were carried out with a Bondmax (Leica Biosystems, Wetzlar, Germany) automated slide staining system, using the Polymer Refine Detection Kit (Menarini Diagnostics, Berlin, Germany). For immunohistochemistry, we used monoclonal mouse antibodies, directed against β-Catenin (clone CAT5H10; Zytomed Systems, Berlin, Germany; dilution 1:300), CDX2 (clone AMT28; Novocastra Laboratories Ltd, Newcastle, United Kingdom; 1:20), chromogranin A (clone LK2H10; Biologo, Kronshagen, Germany; 1:100), CD10 (clone 56C6; Novocastra; 1:10), cytokeratin 7 (CK7; clone RN7; Novocastra; 1:100), cytokeratin 20 (CK20; clone Ks20.8; NeoMarkers, Fremont, United States of America; 1:50), mucin 1 (Muc1; clone Ma695; Novocastra; 1:100), mucin 2 (Muc2; clone Ccp58; Novocastra; 1:100), mucin 5 (Muc5; clone 45M1; NeoMarkers; 1:100), p53 (clone DO-7; Novocastra; 1:100), trefoil factor 2 (TFF2; clone GE16C;

**Table 1**
Immunohistochemical profile of the eight differentiation patterns of the gallbladder mucosa.

| Antigen | Differentiation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Regular gallbladder epithelium | Low grade biliary intraepithelial neoplasia | Papillary adenoma | Paneth cell metaplasia | Goblet cell metaplasia | Pancreatic metaplasia | Pseudopyloric metaplasia | Neuroendocrine differentiation |
| Cytokeratin 7 | + | – | + | + | + | + | – | – |
| Cytokeratin 20 | – | + | – | – | + | – | – | – |
| CDX2 | – | + | – | – | – | – | – | – |
| Mucin 1 | – | – | – | – | – | – | – | – |
| Mucin 2 | – | Single cells + | – | + | + | – | – | – |
| Mucin 5 | – | – | – | – | – | (+) | – | – |
| CD10 | | + | – | – | – | – | – | – |
| Trypsin | – | – | – | – | – | + | – | – |
| β-catenin (nuclear) | – | – | – | – | – | – | – | – |
| p53 | Single cells (+) | Single cells (+) | – | – | – | – | – | – |
| Chromogranin A | – | – | – | – | – | – | – | + |
| Synaptophysin | – | – | – | (+) | – | – | – | + |
| LGR5 | – | – | + | – | – | – | – | – |
| Trefoil factor 2 | – | – | – | – | – | – | + | – |

"+" denotes positivity, "(+)" denotes weak positivity, "–" denotes negativity.

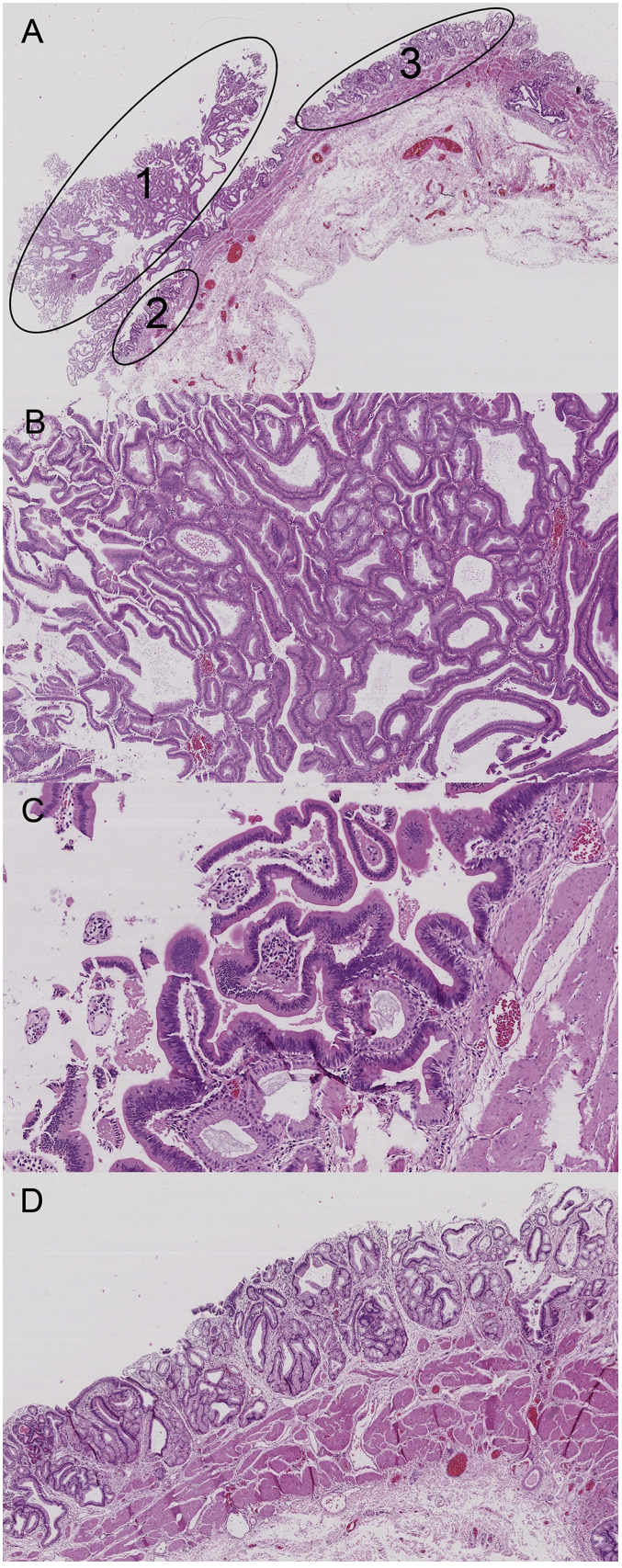


**Fig. 2.** Overview of the gallbladder.
The gallbladder contains a papillary adenoma (A1, B), a low grade biliary intraepithelial neoplasia (A2, C), and regular gallbladder epithelium (A3, D). These three morphologically peculiar areas within the gallbladder mucosa were manually microdissected separately, and mutational analysis of codons 12/13, 61, 117 and 146 of the *KRAS* and *NRAS* genes, codon 600 of the *BRAF* gene, and mutational hotspots in exons 8 and 9 of the *GNAS* gene was performed separately for each phenotype. Hematoxylin and eosin, original magnifications ×5.3 (A); ×50 (B); ×100 (C); ×30 (D).

Leica Biosystems, Newcastle, United Kingdom; 1:10), respectively, monoclonal rabbit antibodies, directed against synaptophysin (clone SP11; NeoMarkers; 1:50), and polyclonal rabbit antibodies, directed against trypsin (BioDesign, Memphis, United States of America; 1:1000) and LGR5 (LGR5-11b; 1:1000) [3]. Pretreatment was done with ER2 (anti-CDX2, anti-β-Catenin) or ER1 (anti-CD10, anti-CK7, anti-CK20, anti-LGR5, anti-Muc1, anti-Muc2, anti-p53, anti-synaptophysin, anti-TFF2) for 20 min. No pretreatment was done for anti-chromogranin A, anti-Muc5, and anti-trypsin. All antibodies were diluted in antibody diluent (Zytomed Systems, Berlin, Germany).

### *Microdissection and DNA extraction*

Three morphologically peculiar areas within the gallbladder (see below in *Molecular findings*) were manually microdissected separately. Genomic DNA was extracted from formalin-fixed and paraffin-embedded tissue using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

### *Mutational analysis*

Mutational analysis of codons 12/13, 61, 117 and 146 of the *KRAS* and *NRAS* genes, codon 600 of the *BRAF* gene, and mutational hotspots in exons 8 and 9 of the *GNAS* gene were performed by pyrosequencing on a PyroMark Q24 instrument (Qiagen). Fragments of the different genes were amplified by polymerase chain reaction.

## Results

### *Histopathologic findings*

After complete embedding of the cholecystectomy specimen, we found eight differentiation patterns within the mucosa, which were: (1) regular gallbladder epithelium, (2) low grade biliary intraepithelial neoplasia (BilIN-1), (3) papillary adenoma with intermediate grade dysplasia, (4) Paneth cell metaplasia, (5) goblet cell metaplasia, (6) pancreatic metaplasia, (7) pseudopyloric metaplasia, and (8) neuroendocrine differentiation. The light microscopic findings of the various phenotypes were confirmed by immunohistochemistry (Table 1, Fig. 1). No relevant inflammatory infiltrate, respectively, chronic inflammatory changes were observed.

Additionally, we explored three other, previously examined gallbladder specimens of FAP patients from the archive of the Institute of Pathology, University Hospital Kiel. These cases showed neither preneoplastic nor neoplastic changes of the mucosa.

### *Molecular findings*

On the basis of the morphologic and immunohistochemical findings, we performed mutational analysis of codons 12/13, 61, 117 and 146 of the *KRAS* and *NRAS* genes, codon 600 of the *BRAF* gene, and mutational hotspots in exons 8 and 9 of the *GNAS* gene of three distinct phenotypic areas (Fig. 2). Area 1 contained the papillary adenoma with intermediate grade dysplasia and showed a gene mutation of codon 12/13 of the *KRAS* gene (c.34G > T; p.G12C) which results in an amino acid substitution at position 12 in KRAS-protein, from a glycine to a cysteine. Area 2 (BilIN1) and area 3 (regular

gallbladder epithelium) showed no mutation within the examined codons and exons of *KRAS*, *NRAS*, *BRAF* or *GNAS*.

## Discussion

We herein report the first case of a complex *APC* germline mutation-associated metaplasia and intraepithelial neoplasia of the gallbladder in a patient with an attenuated FAP. Dysplastic changes, adenomas and carcinomas of the gallbladder in patients with an *APC* germline mutation are – although extremely rare – already known, but to date, nothing has been described about complex synchronous metaplastic *and* dysplastic changes of the gallbladder mucosa of these patients, and nothing is known about these changes in patients with attenuated FAP. In general, FAP patients are at increased risk of developing other types of cancer and mucosal alterations, such as fundic gland polyps and cancer of the stomach, as well as cancer of the small intestine [4]. However, a complex metaplasia as described here has not been found at any other organ site, e.g. stomach, small and large intestine, leading to the conjecture that the gallbladder provides a unique environment for metaplasia and dysplasia. At least 40% of the patients with FAP, who underwent cholecystectomy, are known to have epithelial dysplasia of the gallbladder [5], which might be related to a higher biliary bile acid concentration: The bile of patients with FAP is described to have a greater proportion of deoxycholic acid than the bile of patients without FAP [6]. Bile acids influence, e.g. the cellular proliferation and differentiation of colorectal epithelial cells, and may also be relevant for the development of biliary dysplasia [7].

In routine cholecystectomy specimens, metaplastic changes of the mucosa are frequent but never of this complex phenotype. In general, they seem to be associated with patient gender and age and are considered to be precursor lesions of dysplastic changes of the gallbladder mucosa [8]. In the gallbladder, adenoma and dysplasia are regarded as distinct lesions that are both associated with a highly increased risk for carcinoma development [9]. In this regard, incidental adenomas as precursor lesions for gallbladder adenocarcinoma are rare, whereas the metaplasia → dysplasia → carcinoma in situ → invasive gallbladder carcinoma sequence is much more prevalent and well-established in the concept of gallbladder cancer [10].

Several gene mutations, including mutations within the WNT-signaling pathway, *KRAS*, *BRAF* and *TP53*, are known to play a role in gallbladder carcinogenesis [11]. Within the WNT-signaling pathway, APC is closely connected to β-catenin. In non-neoplastic gallbladder epithelium, the β-catenin expression is solely membranous, whereas a nuclear expression comes along with β-catenin mutations, as they are known to appear in neoplastic changes. All eight epithelial phenotypes found in the gallbladder mucosa of our patient showed a moderate to strong membranous β-catenin expression, whereas they lacked a nuclear β-catenin expression. This observation confirms previous findings, which state that hyperplastic and dysplastic gallbladder epithelium shows a membranous β-catenin expression, whereas a nuclear or cytoplasmic accumulation is mainly reserved to gallbladder carcinomas [2,12]. Thus, the presence of a *β-catenin* mutation may not be present in our patient. *KRAS* gene mutations are known to be involved in the development of gallbladder adenoma, dysplasia and carcinoma [13]. In contrast to the minor role that adenomas play in the carcinogenesis of sporadic gallbladder carcinoma, the combination of a gallbladder adenoma with a *KRAS* mutation in a patient with an *APC* germline mutation may have further interesting implications:

(1) In colorectal adenoma, an APC loss results in the initiation of an adenoma, whereas the progression to a carcinoma needs a second hit, as it is fulfilled, e.g. by a *KRAS* mutation [14]. As this is the first case of a *KRAS* mutation in a gallbladder adenoma of a patient with an *APC* germline mutation, this case may provide evidence that a similar sequence of events may also initiate gallbladder cancer.

(2) The combination of a *KRAS*-mutation of a gallbladder adenoma against the background of an *APC* germline mutation is highly suggestive of an early event of malignant transformation. This conclusion is moreover supported by the proven LGR5 overexpression within the adenoma, which is known to be associated with cancer progression in other gastrointestinal tumor entities [3,15].

## Conclusion

We herein report on a unique case of complex metaplastic and dysplastic changes of the gallbladder and, moreover, the first case of a *KRAS* mutation in a gallbladder adenoma of a patient with an *APC*-germline mutation. The combination of our findings is highly suggestive of an early event of malignant transformation. As a consequence of our findings, clinicians should draw special attention to the gallbladder of FAP patients, and a simultaneous protective cholecystectomy of FAP patients, which undergo colectomy and show conspicuous changes of the gallbladder mucosa, should be performed in these patients in order to eliminate the risk of a synchronous or metachronous gallbladder neoplasia.

## Informed consent

Ethical approval was obtained from the local ethical review board (D 453/10).

## Conflict of interest

The authors declare that no conflicts of interest exist.

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